# Exploring the Effect of PRMT5 Inhibition on the Intrinsic Apoptotic Pathway in Mantle Cell Lymphoma

UNDERGRADUATE RESEARCH THESIS

Presented in Partial Fulfillment of the Requirements for Graduation "with Research Distinction" in the Undergraduate College of Arts and Sciences at The Ohio State University

By

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# Abstract

Mantle Cell Lymphoma (MCL) is an aggressive B cell malignancy that constitutes 3-6% of all Non-Hodgkin's Lymphomas diagnosed annually. MCL is associated with poor prognosis due to resistance to chemotherapies, high incidence of relapse, and late median age of diagnosis. Short of a stem cell transplant, MCL is incurable. Because the average MCL patient is 70 years old at diagnosis, aggressive treatment options are often not realistic. The average survival is five years, and for patients treated with targeted agents such as ibrutinib whose disease progression continues, median survival is a mere three to eight months. This dismal prognosis and lack of effective long-term treatment options makes the development of novel therapeutic approaches for MCL a crucial area of research.

Our group was the first to identify the overexpression of Protein Arginine Methyltransferase 5 (PRMT5) as a key driver of MCL pathogenesis. PRMT5 is an enzyme that catalyzes the methylation of arginine residues on many proteins, including histones. This post-translational modification results in the silencing of tumor suppressor genes which contributes to cell transformation and cancer. Selectively inhibiting PRMT5 significantly reduces tumor burden and delays cancer progression in MCL. The Baiocchi group at The Ohio State University discovered a first in class PRMT5 inhibitor, and in collaboration with Prelude Therapeutics has opened a phase one clinical trial delivering a novel PRMT5 inhibitor to cancer patients at the James Cancer Center and across the country.

In our work to explore the effects of PRMT5 inhibition and its potential to reprogram MCL cells, we have discovered that combining PRMT5 inhibition with BCL-2 inhibition results in synergistic cell death. BCL-2 is one of several members of the BCL-2 family of proteins, which regulate apoptosis at the outer mitochondrial membrane. BCL-2 family proteins can be broadly categorized as pro- or anti-apoptotic and their relative abundances and binding interactions determines if a cell will live or die. The synergy observed between PRMT5 inhibition and BCL-2 inhibition made us curious about what other effects PRMT5 inhibition might be having on the interactions at the outer mitochondrial membrane.

**We hypothesized that PRMT5 inhibition lowers the apoptotic threshold in MCL by modulating levels of BCL-2 family proteins to favor a pro-apoptotic state.** To investigate this, we used two techniques: western blotting to quantify protein concentrations, and BH3 profiling to determine the apoptotic threshold in response to pro-apoptotic stimuli. These results were used to deduce each cell line's dependence on the different anti-apoptotic proteins for survival. BH3 profiling was also performed in PRMT5-inhibited cells to determine if the apoptotic threshold had been altered.

Our results show that at baseline, each MCL cell line exhibited a unique BH3 profile, meaning they responded differently overall when challenged with various BH3 mimetic peptides. Western blotting revealed no marked changes in abundance of pro- or anti-apoptotic proteins with PRMT5 inhibition. We observed that four out of the six cell lines responded to PRMT5 inhibition by exhibiting a lowered apoptotic threshold for at least one of the BH3 peptides. However one of the cell lines, Jeko, exhibited the opposite effect: PRMT5 inhibited cells underwent less mitochondrial depolarization, which may exemplify this cell line's resistance to PRMT5 inhibition. This finding emphasizes the heterogeneity of MCL cell lines and demonstrates how the intrinsic apoptotic pathway can be differentially regulated in similar *in vitro* models.

## Background

Mantle Cell Lymphoma (MCL) is a rare and aggressive B-cell malignancy which constitutes 3-6% of Non-Hodgkin's Lymphomas diagnosed each year [1]. MCL oncogenesis is initiated by chromosomal translocation t(11;14)(q13;32), which places the cell cycle regulator cyclin D1 under control of the IGH heavy chain gene locus. This leads to aberrant CCND1 overexpression and dysregulation of the cell cycle, resulting in proliferative growth [2]. There are four cytologic variants of MCL: classical, small cell, blastoid, and pleomorphic, and the latter two are particularly aggressive [3]. Disease in MCL presents in the lymph nodes, spleen, blood, and bone marrow. MCL is associated with poor prognosis due to many factors, including late median age of diagnosis, resistance to frontline chemotherapies, and high incidence of relapse [4, 5]. Because the average MCL patient is 70 years old at diagnosis, aggressive treatment options such as stem cell transplantation are often not realistic. The average survival rate is five years, and for patients treated with targeted agents such as ibrutinib whose disease progression continues, median survival is just three-eight months [5, 6]. This dismal prognosis and lack of effective long term treatment options makes the development of novel therapeutic strategies for treating of MCL a crucial area of research.

The Baiocchi Group at Ohio State University was the first to identify the overexpression of protein arginine methyltransferase 5 (PRMT5) as a key driver in MCL pathogenesis [7-9]. PRMT5 catalyzes the methylation of arginine residues onto target proteins, including histones, which can have considerable effects on gene expression. As a type-II protein arginine methyltransferase, PRMT5 symmetrically dimethylates the two terminal guanidino groups of the arginine side chain, which interferes with many protein-protein, protein-DNA, and protein-RNA interactions [10, 11]. In particular, PRMT5 symmetrically dimethylates four arginine residues on three core histones: residue 3 on histone four, residues 2 and 8 on histone three, and residue three on histone 2A. Symmetric dimethylation of H4R3 in conjunction with H3R8 is associated with gene silencing in lymphoid cells and lymphomagenesis [12]. PRMT5 is induced translationally by miR-92 and miR-96, and the aberrant expression of these micro-RNAs results in global methylation of histones H3R8 and H4R3 [13]. Dysregulation of PRMT5 is also caused by downstream effectors in the B-Cell Receptor (BCR) signaling pathway, and constitutive activation of this pathway results in increased cell survival and tumorigenesis [14]. Selectively inhibiting PRMT5 has been shown to significantly reduce tumor burden and delay cancer progression in preclinical models of MCL [15, 16]. In collaboration with Prelude Therapeutics, the Baiocchi Group has developed a first-in-class PRMT5 inhibitor which is currently being delivered to patients in a Phase 1 clinical trial at the James Cancer Center and across the country [17] (NCT03886831).

Although PRMT5 inhibition does effectively reduce cancerous growth and cell proliferation, it is not entirely curative. Because of this, and the growing understanding in cancer biology that single-agent treatments are not likely to be curative, we searched for a rational combination treatment by screening several FDA-approved targeted agents. We found that

venetoclax, a selective BCL-2 inhibitor, exhibits significant synergistic killing when combined with PRT-382, a selective PRMT5 inhibitor [16]. Synergistic killing can be described as a greater-than-additive effect, whereby the observed percentage of cells killed when these selective inhibitors are combined is higher than the sum of the percentages of cells killed when these inhibitors act independently. Venetoclax acts by interfering with the binding interaction between two BCL-2 family proteins, BAX and BCL-2. Members of this protein family regulate intrinsic apoptosis at the outer mitochondrial membrane through a dynamic and complex set of binding interactions (Figure 1). BCL-2 promotes cell survival by sequestering BAX, which normally initiates apoptosis upon receiving pro-death signals. Venetoclax competitively binds BCL-2, which releases BAX to assemble homo-oligomers and form a pore in the outer mitochondrial membrane. This triggers membrane permeabilization, cytochrome C release, and caspase 9 activation, which ultimately results in apoptosis of the cell. The observed synergy between venetoclax and PRT-382 motivated us to investigate other potential targets at the outer mitochondrial membrane.

Venetoclax effectively shifts the balance towards the apoptotic threshold in MCL, but it acts at just one of many dynamic binding interactions that regulate intrinsic apoptosis. These binding interactions are mainly between members of the BCL-2 family of proteins. These proteins can be broadly categorized into two groups: pro-apoptotic proteins, which promote and initiate cell death, and anti-apoptotic proteins, which act in favor of cell survival. Pro-apoptotic proteins can be categorized further into activators, sensitizers, and effectors (Figure 1). Activators, such as Bid or Bim, signal for the activation of effector proteins, BAK or BAX, which homo-oligomerize to form a pore in the mitochondrial outer membrane. This initiates the release of cytochrome c and ultimately triggers apoptosis. Sensitizers act by competitively binding anti-apoptotic proteins, which prevents anti-apoptotic proteins from sequestering activators/effectors and sensitizes cells to the initiation of apoptosis.

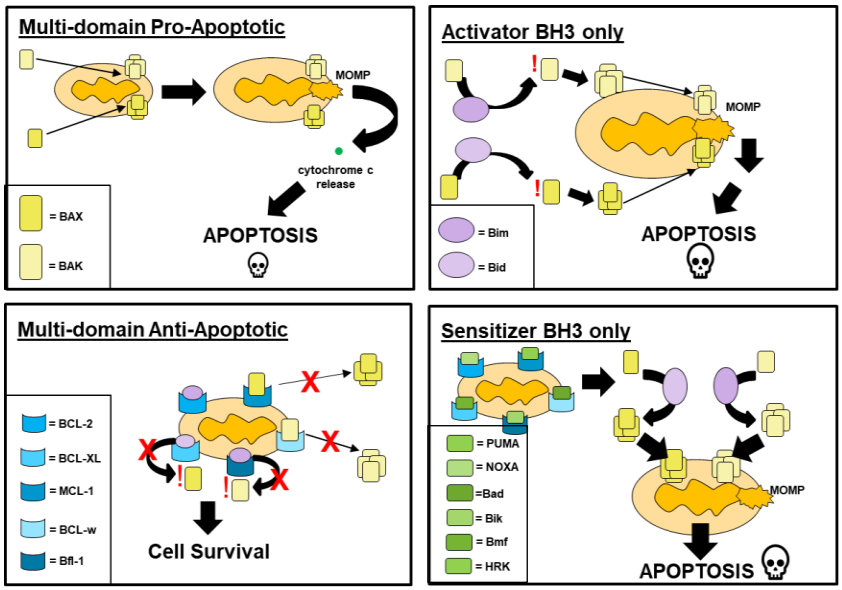



*Figure 1: A diagram of the four classes of BCL2 family proteins. TL: Pro-apoptotic effectors which are able to cause apoptosis through homo-oligomerization and pore assembly. The pore then depolarizes the mitochondria and releases cytochrome C. TR: Activator proteins facilitate the binding of pro-apoptotic effector proteins to push towards apoptosis. BL: Anti-apoptotic proteins have the ability to sequester both pro-apoptotic effectors and activators. They are pro-survival proteins and are commonly upregulated in cancers. BR: Sensitizer proteins bind competitively to the anti-apoptotic proteins allowing activators and effectors to interact.*

To add another layer of complexity to these interactions, not all pro- and anti-apoptotic proteins bind with the same affinity [18] (Figure 2). For example, although pro-apoptotic activators Bid and Bim can be sequestered by every anti-apoptotic protein, Bim associates with

BCL-2 much more readily than Bid does. Because of unique binding affinities such as these, it is insufficient to come up with a sum of all pro- and anti-apoptotic proteins to predict the outcome of a death insult. Luckily, these binding interactions have been well-characterized and can be used to inform the extent to which a cell has been primed for apoptosis, as well as the identity of anti-apoptotic proteins most critical for survival. We sought to better understand the biology of MCL by characterizing the upstream events that regulate intrinsic apoptosis at the outer mitochondrial membrane. We also wanted to explore the effects of PRMT5 inhibition on the intrinsic apoptotic pathway in hopes of either elucidating the mechanism of synergy between venetoclax and PRT-382, or identifying novel targets for combination therapies. This was achieved through an assay called BH3 profiling. BH3 profiling allows us to determine the extent to which a cell has committed to apoptosis without needing to characterize each and every binding interaction taking place, and it also reveals the identity anti-apoptotic proteins that are most crucial in the prevention of MOMP. Mitochondrial outer membrane permeabilization is irreversible and marks the initiation of apoptosis, so defining the dynamic interactions of BCL-2 family proteins and determining the apoptotic threshold of MCL cells could provide insight into MCL biology as well as potential therapeutic strategies.

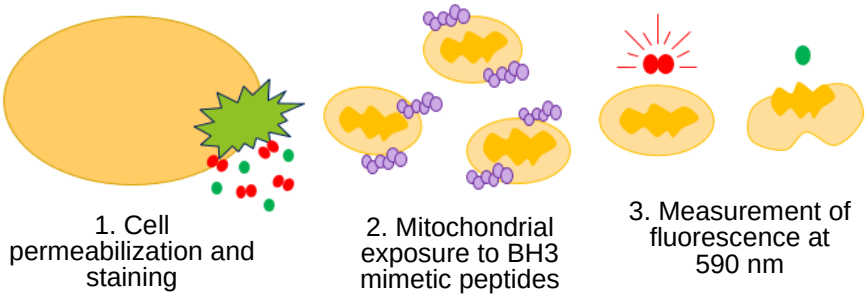
| | BID | BIM | BIDM | BAD | BIK | NOXA A | NOXA B | HRK | BNIP | PUMA | BMF |
|---|---|---|---|---|---|---|---|---|---|---|---|
| BCL2 | 66 (6) | <10 | - | 11 (3) | 151 (2) | - | - | - | - | 18 (1) | 24 (1) |
| BCLXL | 12 (9) | <10 | - | <10 | 10 (2) | - | - | 92 (11) | - | <10 | <10 |
| BCLW | <10 | 38 (7) | - | 60 (19) | 17 (12) | - | - | - | - | 25 (12) | 11 (3) |
| MCL1 | <10 | <10 | - | - | 109 (33) | 19 (2) | 28 (3) | - | - | <10 | 23 (2) |
| BFL1 | 53 (3) | 73 (3) | - | - | - | - | - | - | - | 59 (11) | - |

*Figure 2: Binding affinities of BH3-only peptides to N-terminal GST, C-terminal truncated anti-apoptotic proteins. Values are EC50 in nM concentrations. Modified from "A Laboratory Guide to BH3 Profiling" Letai et al.*

# Methods

## JC-1 Plate-Based BH3 Profiling

This assay was created by the Letai lab in order to determine a cell's net response to pro-apoptotic stimuli through BH3 mimetic peptides [19]. These mimetic peptides are truncated versions of the BH3 only activators and sensitizers, all of which interact with anti-apoptotic




proteins with different affinities. The general workflow is as follows:

1. Cell Permeabilization and staining: Cells were resuspended in the JC-1 buffer containing a pore-forming detergent, digitonin, to maintain cell viability while allowing the peptides to

pass through the cell membrane. The buffer also contained the lipophilic cationic dye, JC-1, which aggregates in active, polarized mitochondria. JC-1 emits light at two different wavelengths in its aggregate vs. monomeric form, which allows for the determination of mitochondrial activity. Cell suspension was optimized to 3e6/ml which was plated in black 384 well plates with minimal light exposure under sterile conditions.

2. Exposure to BH3 mimetic peptides: BH3 peptide mimetics were purchased from Aapptec as described by the Letai protocol. Peptide salts were stored at -20 degrees Celsius and then resuspended at 10mM in DMSO. Working aliquots were stored at -20 degrees Celsius and the stock stored at -80 degrees Celsius. Dilutions from the working aliquot were made into MEB to reduce the concentration of DMSO. 2X peptide stocks were made and then plated with the cells.
3. Measurement of Fluorescence at 590 nm as a Surrogate of MOMP: The plate was spun down at 600G for 30 seconds to ensure all reagents were at the bottom of the wells. A plate reader with a monochromatic filter to allow excitation of only the red JC-1 aggregates was used to monitor relative fluorescent unites (RFU) of each well at 5 minute intervals for 150 minutes. Kinetic traces were created by plotting RFU at 590 nm over time, normalized to the highest NT value. The area under the curve of each kinetic trace was used to synthesize a profile depicting the net response to each peptide, which was normalized to DMSO.

Cell Culture Work

Six cell lines were used in this work: Jeko, UPN-1, Z-138, Mino, Maver-1, and Granta-519. All lines were cultured at 37 degrees Celsius, 5% CO2, in RPMI 1640 supplemented with 10% FBS (Sigma Aldrich #F4135), 1% glutamax (Gibco, Thermo Fisher #35050-061), and 1% penicillin/streptomycin (Gibco #15140-122). Cell lines were validated by STR typing within one year of data collection. Mycoplasma testing (Lonza #LT07-518) was performed monthly.

Drugging

PRT382 was generously supplied by Prelude Therapeutics. To administer drug *in vitro*, cells were seeded at 0.3e6 cells/ml in fresh full RPMI media. PRT382 was solubilized at 100mM in DMSO (Fisher Chemical #D128-500) and log fold dilutions in DMSO were made. These were stored at -20 degrees Celsius for no longer than one month or ten freeze thaw cycles, whichever came first. No more than 0.1% DMSO v/v was added to a given culture. Approximate IC50 of PRT382 at day nine as determined by single agent IC50 studies [Appendix 1], were used prior to BH3 profiling. For six day experiments, cells were resuspended in fresh drugged media at 0.3e6 cell/ml every three days.

Western Blotting

Cells were cultured and drugged as described above. Viability was collected on the day of collection as well as on days the culture was refed using trypan blue and a hemocytometer. At least 5e6 cells were collected for western blotting. The calculated quantity of media for western blots was centrifuged at 1250 rpm for 8 minutes. The pellet was aspirated as dry as possible and then re-suspended in 1 ml of ice cold PBS and transferred to a microcentrifuge

tube. The cells were re-pelleted at 6785g at 4 degrees Celsius for 10 minutes. These were aspirated dry and stored at -80 degrees Celsius for later use.
Dry pellets were lysed in RIPA buffer with phosphatase and protease inhibition cocktails using approximately 80ul per 10e6 cells. Samples were vortexed vigorously every 10 minutes for 30 minutes, incubating on ice between mixing. These were then pelleted at max speed (~13200 g) in a microcentrifuge at 4 degrees Celsius and the supernatant transferred to a new microcentrifuge tube. The quantity of protein in each sample was determined by BCA using the supplied protocol. Between 20 to 30 micrograms of protein were separated on a 4-20% SDS-PAGE gel using between 80 and 120 volts in TGS. Proteins were transferred onto polyvinylidene difluoride (PVDF) membrane using the semi-dry transfer system and Turbo transfer blotting buffer. After transfer, the membranes were briefly rinsed in DI water to remove residual salts and allowed to fully dry. Blots were reactivated in methanol and blocked in LiCor Odessy Blocking Buffer for at least one hour, at room temperature with constant rocking. Primary antibodies were used at a 1:1000 dilution in TBS-t (0.01% tween) with the exception of GAPDH which was used at a 1:3000 dilution. Probing was completed overnight at 4 degrees Celsius with constant rocking. Blots were then washed with TBS-t for 10 minutes three times, before being blocked with LiCor fluorescent secondary antibodies. These antibodies were used at a 1:20,000 dilution in TBS-t for at least one hour at room temperature with constant rocking. A second 30 minute wash with TBS-t was performed as before. The blots were detected on a CLX licor scanner. ImageStudio was used to optimize images as well as quantify bands. Bands of interest were normalized against GAPDH.

Data Analysis and Statistics

Kinetic traces and AUTC values were exported from Gen5 and imported to excel. Data was normalized to the last value of the ‘no treatment’ category for kinetic traces and to the DMSO integral value for profiles. Outliers were censored before mean and standard deviation calculations were done. A student’s T-test with three replicates was used to determine significance between any two profiles.

# Results

## I. Baseline Kinetic Traces

### A. Controls for Depolarization

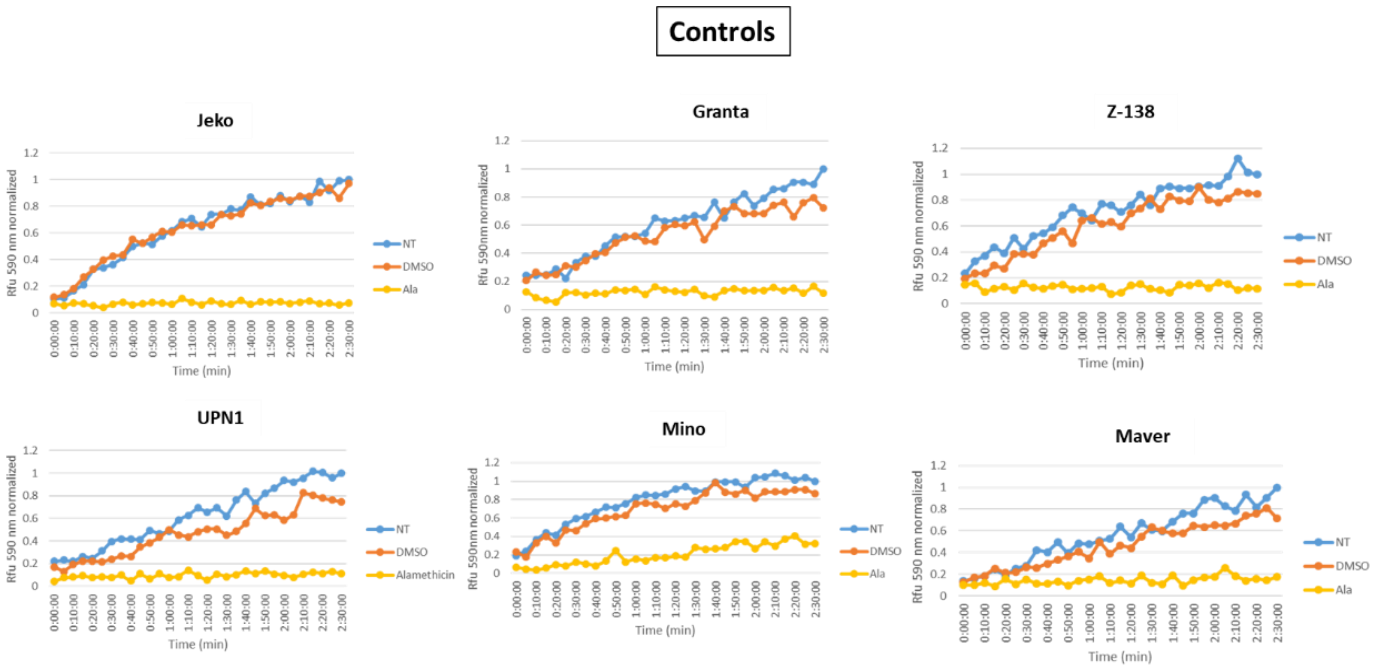
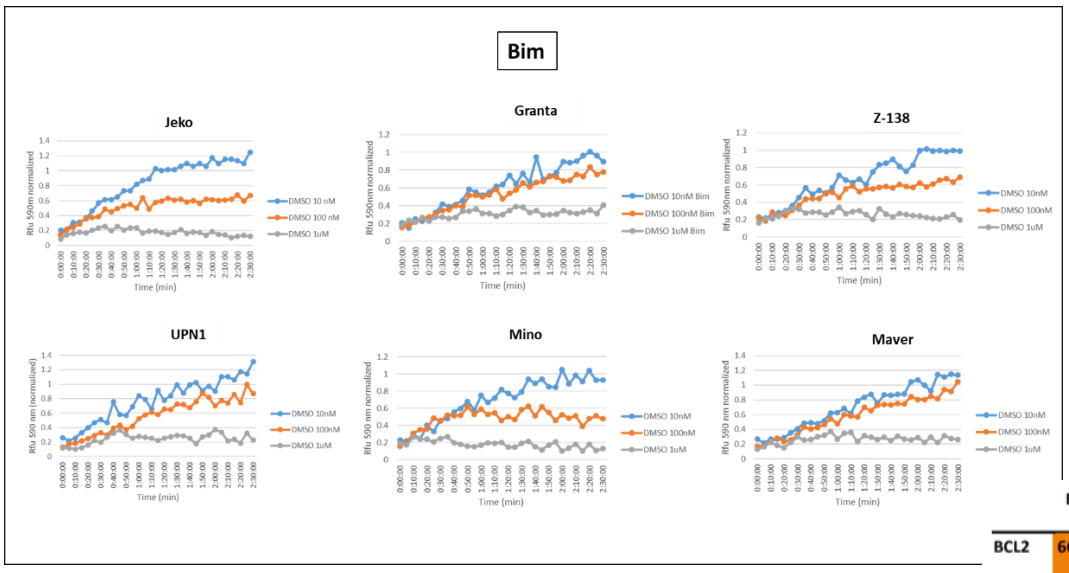



***Figure 3- Controls for Depolarization***. *Untreated cells and DMSO-treated cells were used as negative controls, in which no mitochondrial depolarization occurred, and are depicted by blue and orange traces, respectively. Alamethicin, depicted in yellow, is a pore-forming reagent used as a positive control. All kinetic traces were normalized to the highest NT value and traces portray this normalized value of RFU 590 nm over time.*

## B. Activator Peptides




| | BID | BIM |
|---|---|---|
| BCL2 | 66 (6) | <10 |
| BCLXL | 12 (9) | <10 |
| BCLW | <10 | 38 (7) |
| MCL1 | <10 | <10 |
| BFL1 | 53 (3) | 73 (3) |

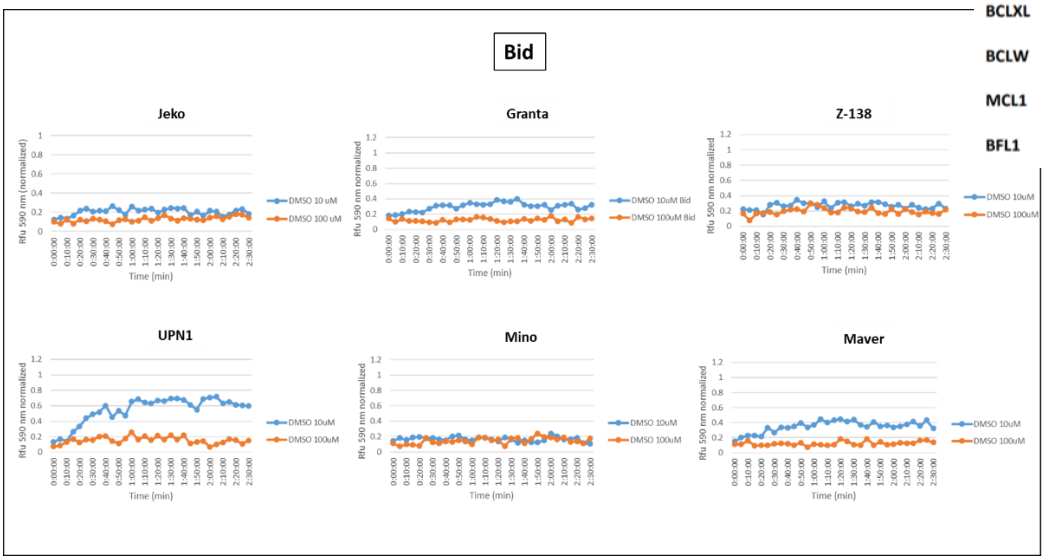



***Figure 4A- Activator Peptide Kinetic Traces.*** *Each cell line was treated with the same peptide concentration: Bim at 10 nM, 100 nM, and 1 uM; and Bid at 10 uM and 100 uM. Bim and Bid interact with effector proteins to initiate oligomerization assembly and pore formation, but they can also be sequestered by anti-apoptotic proteins to prevent MOMP. At high enough peptide concentrations, activators can overcome sequestration and fulfil their duty of activating BAK/BAX.*

## C. Sensitizer Peptides

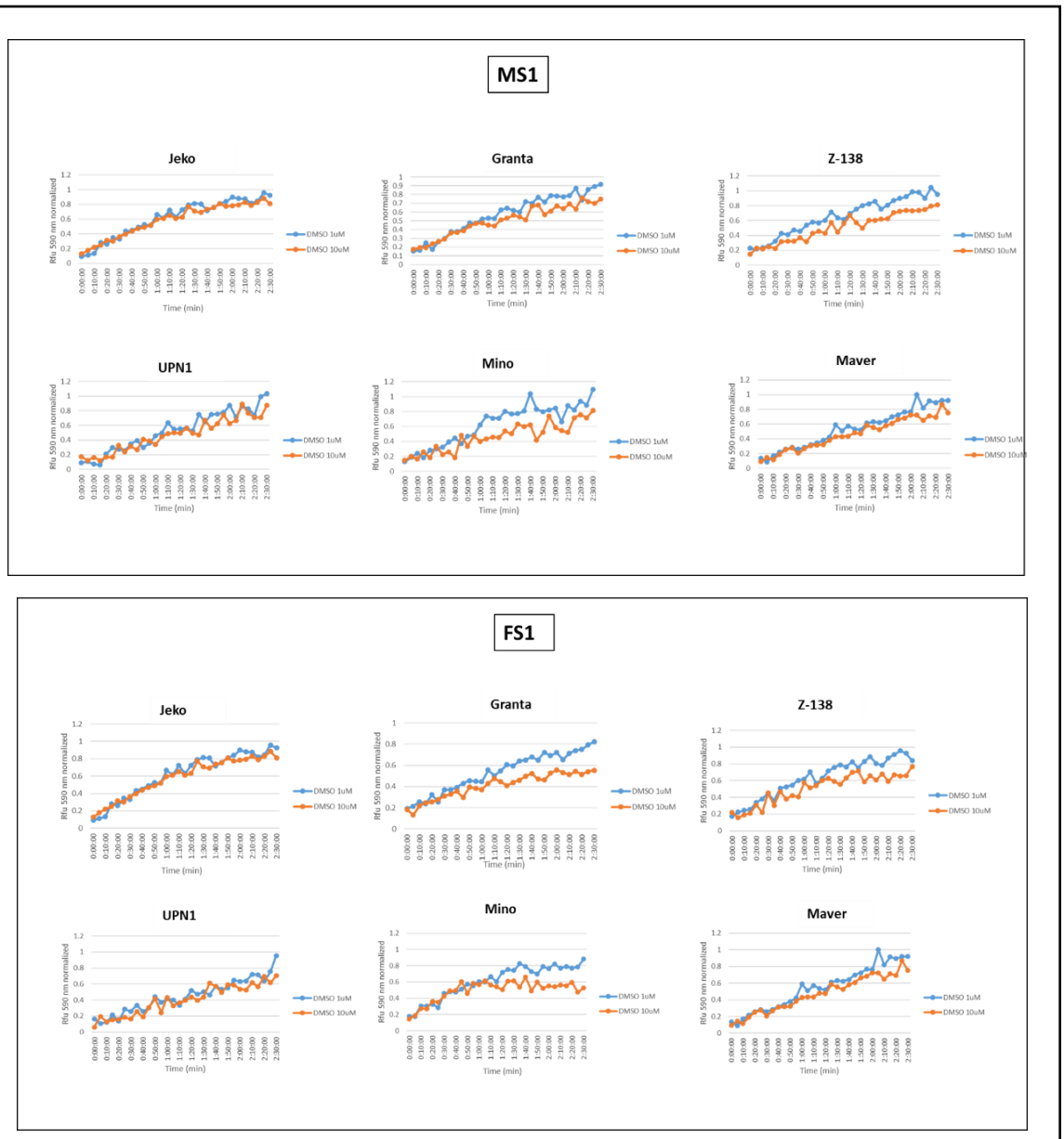
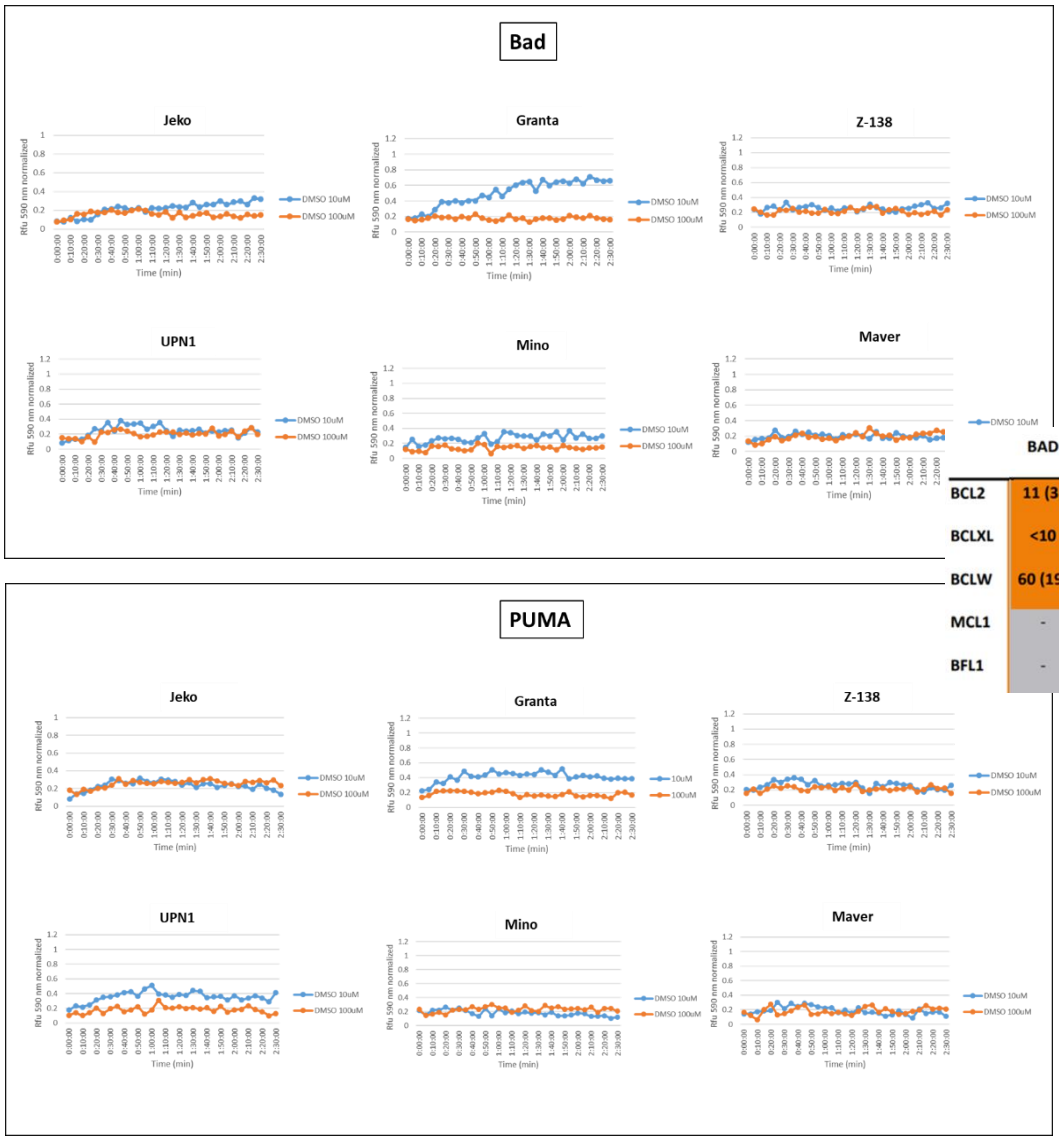



***Figure 4B- Resistant Responses to Sensitizer Peptides*** *Cell lines were treated with the same peptide concentrations for both MS1 and FS1: 1 uM and 10 uM. MS1 and FS1 interact with MCL-1 and Bfl-1, respectively, which sensitizes cells to apoptosis through competitive binding, ultimately preventing sequestration of other pro-apoptotic family members. MS1 and FS1 are not derived from endogenously expressed BH3 proteins.*

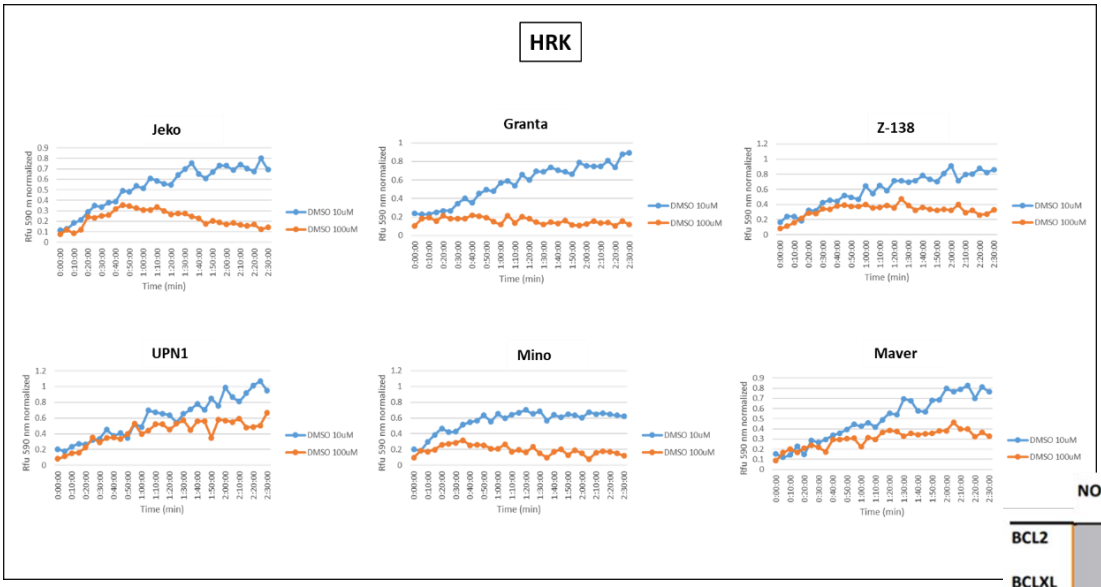
C. Sensitizer Peptides cont.




| | BAD | PUMA |
|---|---|---|
| BCL2 | 11 (3) | 18 (1) |
| BCLXL | <10 | <10 |
| BCLW | 60 (19) | 25 (12) |
| MCL1 | - | <10 |
| BFL1 | - | 59 (11) |

***Figure 4C- Sensitive Responses to Sensitizer Peptides*** *Bad and PUMA were titrated at 10 uM and 100 uM concentrations, and all cell lines exhibited a high degree of depolarization. PUMA is a pan-sensitizer and can bind any anti-apoptotic protein but interacts most closely with BCL-XL, MCL-1, and BCL-2. Bad only binds with three of the five anti-apoptotic proteins: BCL-2, BCL-XL, and Bcl-w.*

C. Sensitizer Peptides cont.




| | NOXA A | NOXA B | HRK |
|---|---|---|---|
| BCL2 | - | - | - |
| BCLXL | - | - | 92 (11) |
| BCLW | - | - | - |
| MCL1 | 19 (2) | 28 (3) | - |
| BFL1 | - | - | - |

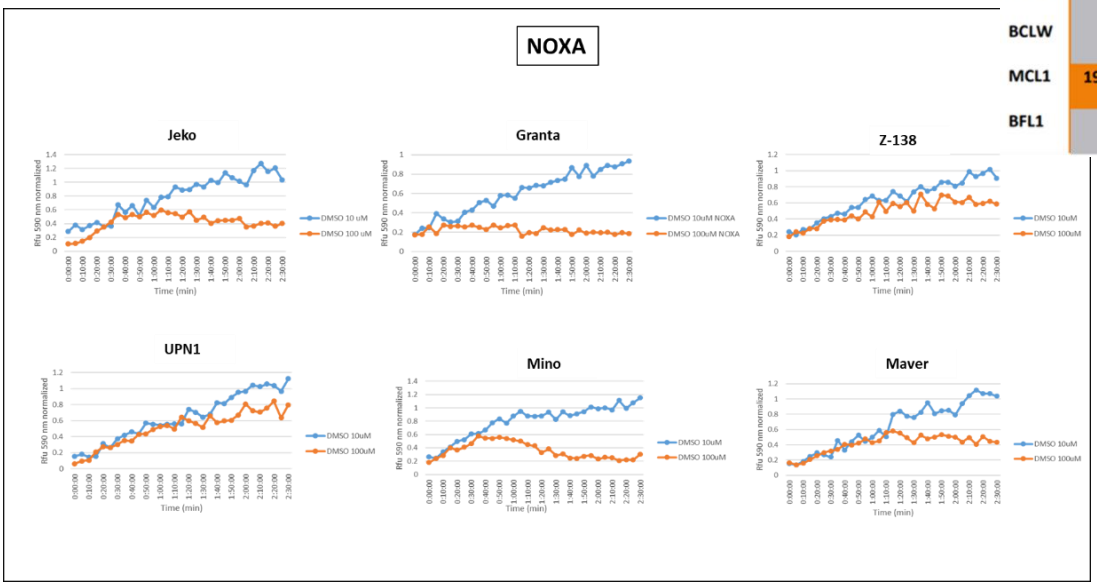
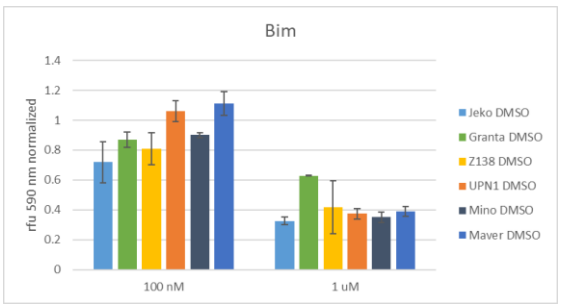



***Figure 4D- Differential Responses to Sensitizer Peptides*** *HRK and NOXA were titrated at 10 uM and 100 uM concentrations, and cell lines exhibited varying degrees of depolarization. NOXA interacts exclusively with MCL-1, and HRK interacts exclusively with BCL-XL, so sensitivity to either of these peptides implicates their respective binding partners as crucial for survival.*

# II. Baseline Profiles

## A. Activator Peptides



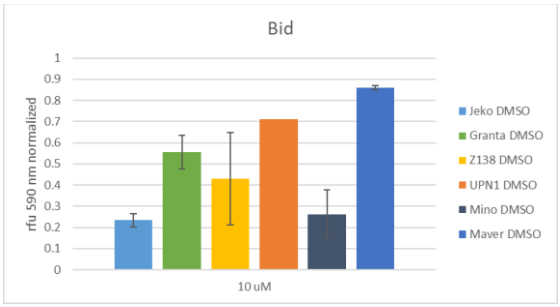
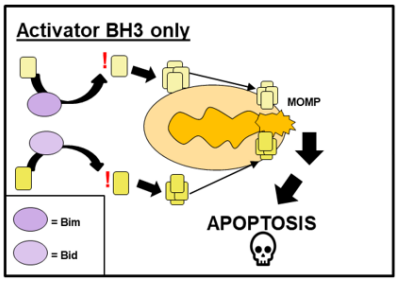
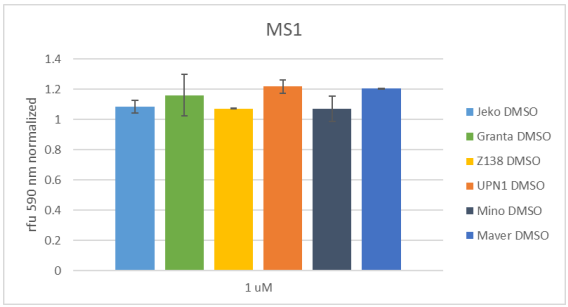




***Figure 5A- Net Responses to Activator Peptides.*** *Profiles are shown for Bim at both 100 nM and 1 uM, and Bid at 10 uM. A dose response pattern exists for Bim but all cell lines tend to respond with a similar degree of mitochondrial depolarization. Bid exhibits differential responses across cell lines.*




## B. Sensitizer Peptides



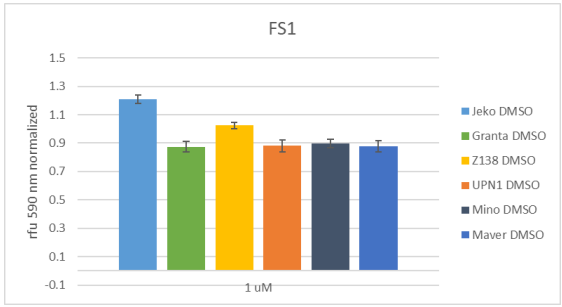
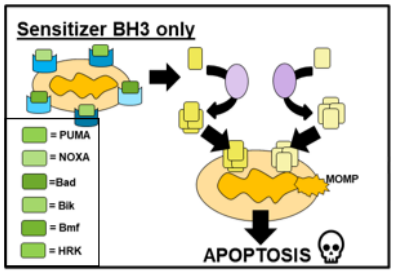
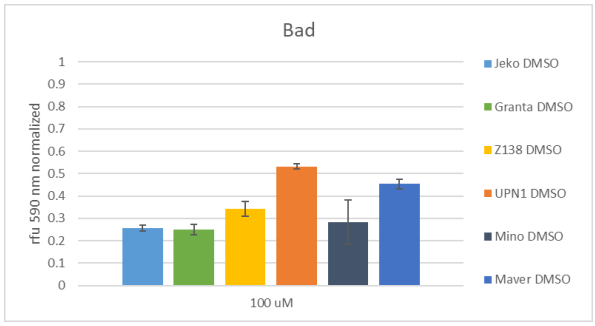




***Figure 5B- Net Responses to Sensitizer Peptides.*** *MS1 and FS1 profiles are reflective of traces and show that very little mitochondrial depolarization is occurring, as indicated by high levels of RFU 590nm. This net response reinforces the widespread resistance to these peptides in MCL cell lines.*



## B. Sensitizer Peptides cont.



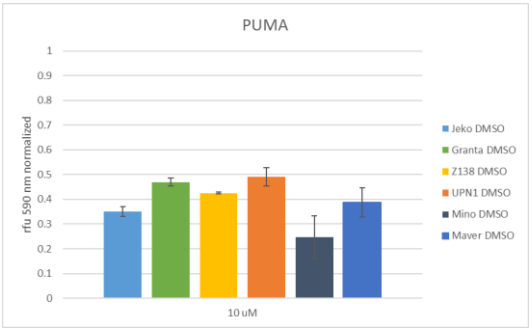
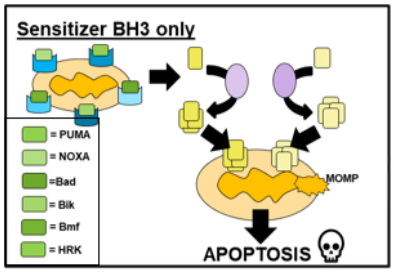
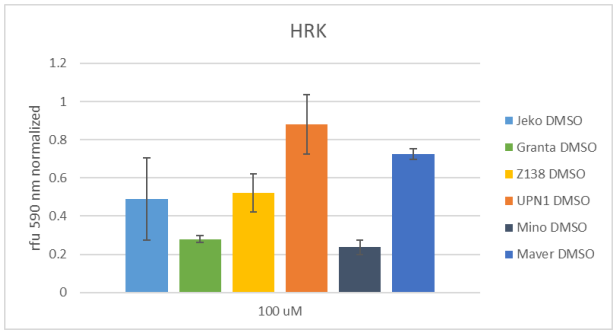




***Figure 5C- Net Responses to Sensitizer Peptides***. *Bad and PUMA profiles reflect the widespread sensitivity of cells treated with these peptides. Both peptides caused a high degree of mitochondrial depolarization as indicated by the low levels of RFU 590 nm (normalized to DMSO).*



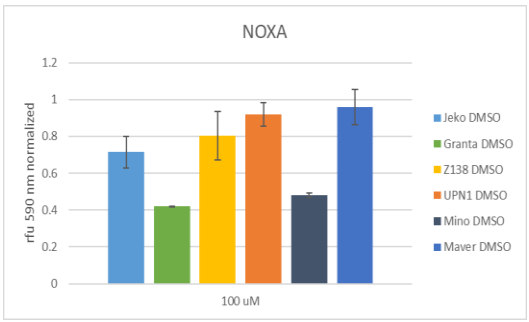
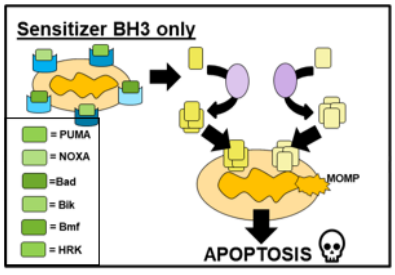




***Figure 5D- Net Responses to Sensitizer Peptides.*** *HRK and NOXA profiles reflect the differential responses exhibited by different cell lines. Granta and Mino were both relatively sensitive to these pro-apoptotic stimuli while the other four cell lines exhibited a degree of resistance at baseline. All baseline profiles were created from DMSO control cells.*



## III. Traces and Profiles Showing Threshold Shift with PRMT5 Inhibition

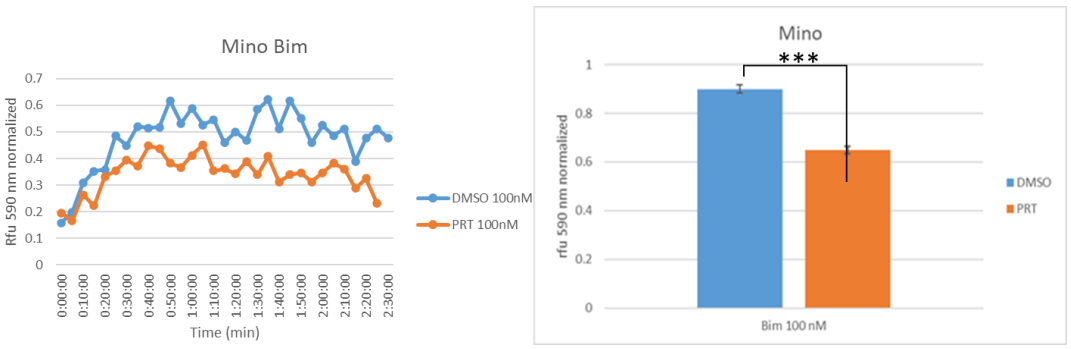



***Figure 6A- Responsiveness of Mino to PRMT5 inhibition*** *Mino exhibited a lowered apoptotic threshold in PRMT5-inhibited cells treated with Bim at 100 nM. Kinetic traces and profiles for control cells are shown in blue, and PRT-treated cells are shown in orange. p<0.001*

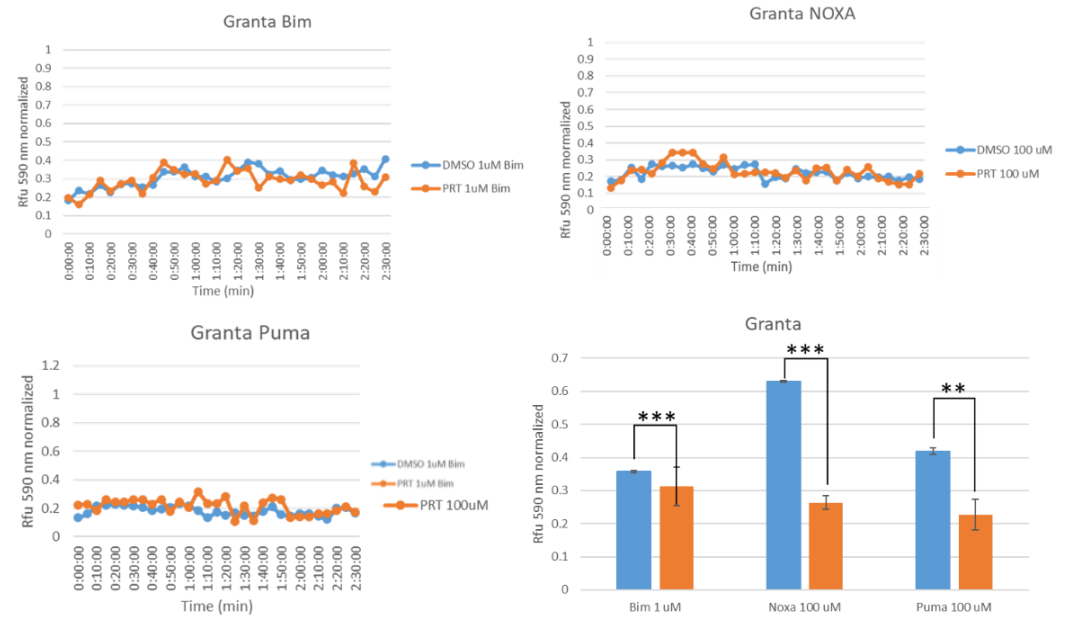



***Figure 6B- Responsiveness of Granta to PRMT5 inhibition*** *Granta exhibited a lowered apoptotic threshold in PRMT5 inhibited cells treated with Bim, Noxa, and Puma. For Bim and Noxa, p<0.001, and for Puma, p<0.01.*

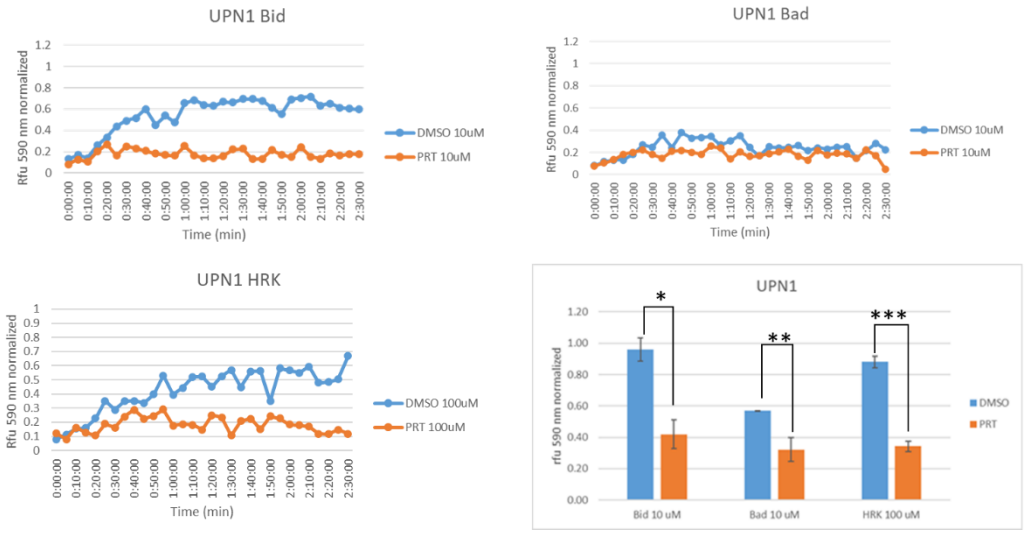



***Figure 6C- Responsiveness of UPN1 to PRMT5 inhibition*** *UPN1 exhibited a lowered apoptotic threshold in PRMT5-inhibited cells treated with Bid and Bad at 10 uM, and HRK at 100 uM. For Bid, p<0.05, for Bad p<0.01, and for HRK p<0.001.*

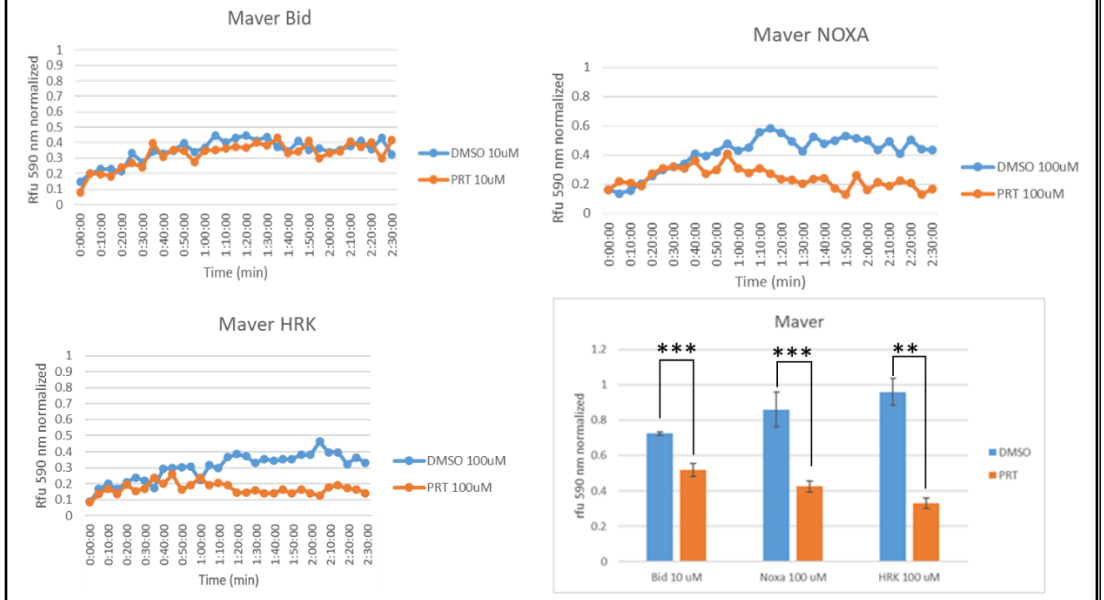



***Figure 6D- Responsiveness of Maver to PRMT5 inhibition*** *Maver exhibited a lowered apoptotic threshold in PRMT5-inhibited cells treated with Bid at 10 uM, and HRK and Noxa at 100 uM. For Bid, p<0.001, for Noxa p<0.001, and for HRK p<0.01.*


***Figure 6E- Responsiveness of Jeko to PRMT5 inhibition*** *Jeko exhibited an unexpected trend in which the apoptotic threshold in PRMT5-inhibited was increased compared to control cells. This was exhibited in Bad at 10 uM and Noxa at 100 uM. For Bad, p<0.05, and for Noxa, p<0.001*

## IV. Western Blots of Anti-Apoptotic Proteins

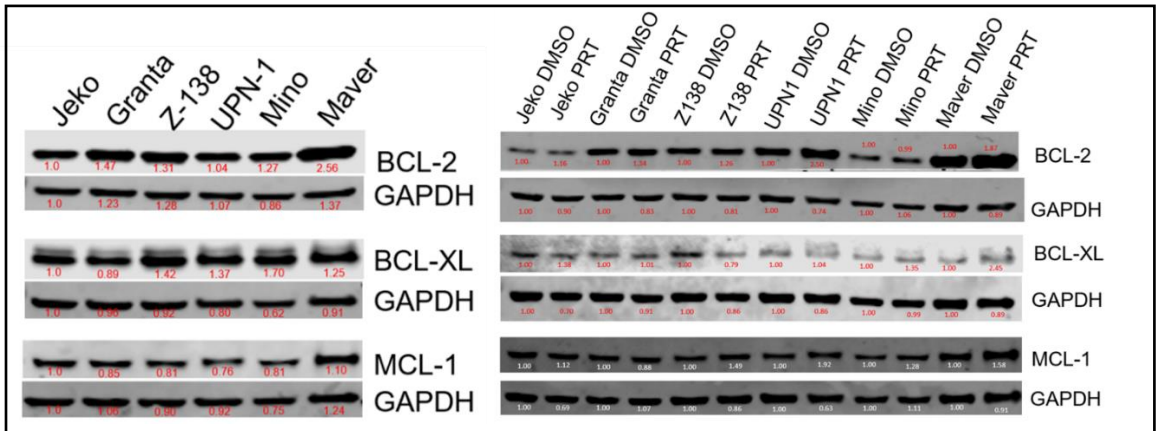



***Figure 7-Expression of Key Anti-Apoptotic Proteins in Normal and PRMT5-Inhibited Cells*** *Western blot analysis revealed increased abundance of BCL-2 and MCL-1 in Maver, suggesting either heightened gene expression or a potential duplication of these genes. Overall, no change in expression levels was observed in control cells compared to PRMT5-inhibited cells for those anti-apoptotic BCL-2 family proteins identified as crucial for survival.*

## V. Summary: Models of Responsiveness

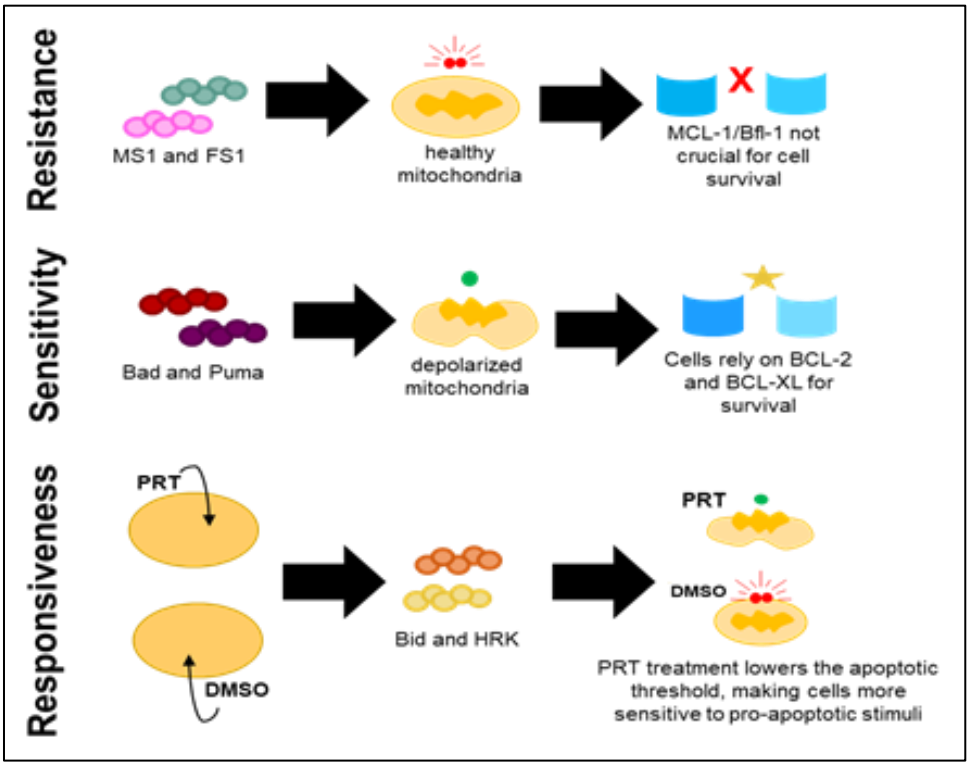



***Figure 8-Classification of Cell Line Response to Peptides*** *All lines were resistant to treatment with MS1 and FS1, exhibiting high levels of RFU 590 nm, indicating either lack of sensitivity to the peptides or lack of reliance on the peptides' binding partners for survival. All lines were sensitive to treatment with Bad, Puma, and Bim peptides, implicating these peptides' binding partners as crucial for preventing apoptosis. Four out of six cell lines were responsive to PRMT5 inhibition and exhibited a lowered apoptotic threshold in PRMT5 inhibited cells compared to control cells, making cells more sensitive to pro-apoptotic stimuli.*

# Discussion

### Binding Profiles

At baseline, there were several peptides that evoked a similar apoptotic response across all six MCL cell lines, including MS1, FS1, Puma, Bad, and Bim. It is apparent that all cell lines are resistant to treatment with the MS1 and FS1 peptides, which are sensitizers designed to bind with MCL1 and Bfl-1, respectively (Figure 4B/5B). If only profiles for MS1 and FS1 were considered, one could postulate that cells are not relying as much on MCL1 or Bfl-1 for survival, because inhibition of these anti-apoptotic proteins through MS1 and FS1 did not result in mitochondrial depolarization. However, cell line responses to other peptides suggest otherwise: all cell lines were sensitive to at least one peptide that closely interacts with MCL1 (Figure 4C), so it is likely that this protein is important for preventing apoptosis. The same cannot be said for Bfl-1, whose only peptide binding partner other than FS1 is Noxa. Treatment with Noxa resulted in a mixed response across cell lines; Granta and Mino were sensitive at baseline, but all other

cell lines were resistant (Figure 4D/5D), suggesting that Bfl-1 is inconsequential in the prevention of apoptosis in these lines.

All cell lines were sensitive to treatment with Puma, Bad, and Bim peptides (Figure 4A/5A, Figure 4C/5C). Puma is a pan-sensitizer, capable of sequestering all anti-apoptotic proteins in support of apoptosis; it makes sense that cells are sensitive to widespread inhibition anti-apoptotic proteins. Bim is also capable of binding to all anti-apoptotic proteins, but in doing so it loses its ability to activate the effector proteins, BAX and BAK. Bim exhibits the tightest binding patterns with BCL-2, BCL-XL, and MCL1, and in the low micro-molar range caused widespread mitochondrial depolarization in all cell lines. It's likely that at this peptide concentration, Bim was in excess and able to fulfil its duty as an activator despite sequestration by its anti-apoptotic binding partners. Unlike Puma and Bim, Bad has a more specific binding pattern; it only interacts with BCL-2, BCL-XL, and Bcl-w. Bcl-w proved difficult to probe for using western blot analysis so relative protein levels between MCL lines was not determined. Bcl-w does not exhibit as tight of binding with Bad as BCL-2 and BCL-XL, so it's likely that BCL-2 and BCL-XL are more crucial than Bcl-w for promoting survival.

Not all cell lines exhibited the same response to peptide treatment. Differential responses were exhibited across cell lines for the peptides Noxa, HRK, and Bid. Noxa caused mitochondrial depolarization in Granta and Mino, but Jeko, Z138, UPN1, and Maver were all resistant to peptide treatment and maintained intact mitochondria (Figure 4D/5D). As a sensitizer, Noxa complexes with MCL-1, reinforcing MCL-1's role in promoting survival in Granta and Mino. Treatment with HRK caused marked depolarization in Jeko, Granta, Z138, and Mino, but UPN1 and Maver exhibited resistance at baseline (Figure 4D/5D). HRK is a sensitizer that binds exclusively to BCL-XL, reinforcing BCL-XL's importance for survival in those lines that were sensitive. Treatment with Bid caused depolarization in Jeko, Z138, UPN1, and Mino, while Granta and Maver exhibited resistance (Figure 4A/5A). Bid, like Bim, is an activator and can be sequestered by all anti-apoptotic proteins, but it interacts with BCL-XL, MCL-1, and Bcl-w with the greatest affinity. This implicates the importance of these three anti-apoptotic proteins in the promotion of cell survival.

**Effects of PRMT5 inhibition on the Apoptotic Threshold**

Four out of six cell lines chosen exhibited a lowered apoptotic threshold in PRMT5 inhibited cells for at least one of the eight peptides. Mino showed a shift in depolarization with Bim peptide treatment (Figure 6A), and Granta exhibited a similar shift at a higher peptide concentration of Bim (Figure 6B). Granta also showed a heightened sensitivity to treatment with Bid in PMRT5 inhibited cells (Figure 6B), as did UPN1 (Figure 6C) and Maver (Figure 6D). UPN1 also exhibited a shift in depolarization with the sensitizers Bad and HRK. Maver exhibited this same trend with HRK, in addition to Noxa. Jeko exhibited a peculiar pattern in which PRMT5 inhibited cells exhibited even less mitochondrial depolarization than control cells when exposed to the peptides Noxa and Bad (Figure 6E). Our previous *in vitro* work has revealed that Jeko is resistant to PRMT5 inhibition, and while it is possible this trend is just an outlier in the data due to small sample size, it also might point towards a mechanism behind resistance to PRMT5 inhibition.

### Priming Class

Results of BH3 profiling can be used to categorize cell lines based on their priming class. Priming refers to how close a cell is to reaching its apoptotic threshold, and based on their response to peptide subtypes, cells can be classified as either Class A, B, or C [20]. The more primed a cell is, the closer it is to undergoing mitochondrial depolarization and committing itself to apoptosis. Class A cells are less primed and respond only to BH3 only activator proteins. These cells are apoptotically competent but generally do not have pre-bound sensitizers, meaning anti-apoptotic proteins are able to sequester activators and effectors. This pushes cells further from reaching their apoptotic threshold. Class B cells are considered apoptotically incompetent because they lack functional BAK and BAX, so no degree of pro-apoptotic stimuli will be capable of initiating mitochondrial outer membrane permeabilization. Class C cells are the most apoptotically primed and respond to both activators and sensitizers. These cells tend to have pre-bound sensitizer proteins, which allows activators and effectors to perform their respective duties for the initiation of apoptosis. Because all six cell lines were responsive to at least one activator peptide and multiple sensitizer peptides, they fall under the Class C category of apoptotic priming and are predicted to be responsive to chemotherapeutic agents that operate as BH3 mimetics. All cells had mixed profiles, meaning they exhibited a dependence on multiple pro-survival BCL-2 family proteins. This finding provides rationale for exploring cell sensitivities to targeted agents that operate at the outer mitochondrial membrane and searching for potential combinational therapies that target multiple anti-apoptotic proteins.

## Conclusion

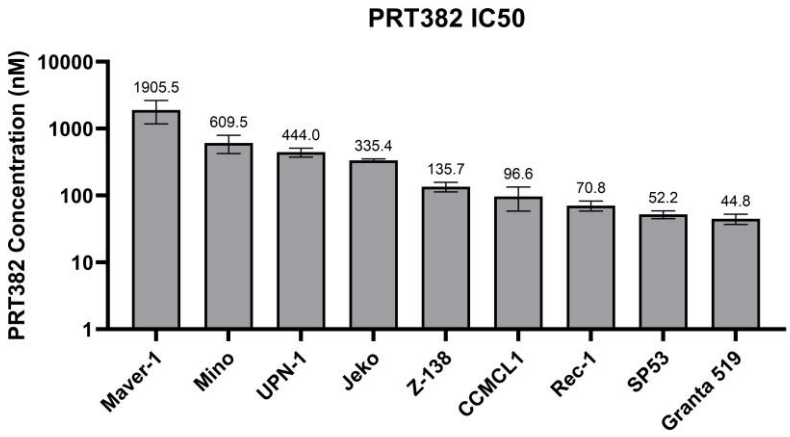
In this work, we have demonstrated that BH3 profiling is a useful and informative technique for characterizing the biology of Mantle Cell Lymphoma at the outer mitochondrial membrane. MCL cell lines exhibited differences in sensitivity to both BH3 only activators and sensitizers, which emphasizes the heterogeneity of these cell lines and exhibits how intrinsic apoptosis can be differentially regulated in similar *in vitro* models. All cell lines fall under the Class C category of apoptotic priming, which means they are naturally primed for apoptosis and will likely exhibit responsiveness to treatment with chemotherapeutic agents targeting anti-apoptotic proteins. These results warrant further research into the effects of BH3 mimetics in MCL including compounds such as navitoclax (ABT-263), a pan-inhibitor of BCL-XL, BCL-2, and Bcl-w, or AMG-176, a selective MCL-1 inhibitor.

While these findings are promising, there are limitations to this work that will be addressed in future studies. For example, all cell lines exhibited high levels of depolarization in both control and PRMT5-inhibited cells in response to Puma, Bad, and Bim. Because of this, we were unable to determine whether or not PRMT5 inhibition shifted the apoptotic threshold under these treatment conditions. Future work will involve exploring more optimal peptide concentrations and strengthening our findings through more replicates. It is also plausible that the peak effect of PRMT5 inhibition on the apoptotic threshold occurs earlier on during the six day drugging with PRT-382, so the maximal effects of PRMT5 inhibition might not have been captured in these experiments. To address this, future studies will be conducted investigating the effects of PRMT5 inhibition at 24 hour time points during the six day drugging period.

Another avenue of this work will be exploring the effects of PRMT5 inhibition on the intrinsic apoptotic pathway in primary patient samples and/or patient derived xenograft cells. Because these samples will involve heterogeneous cell populations, we will be utilizing a flow cytometry based- method called iBH3 profiling in place of JC-1 based BH3 profiling, which is better suited for homogenized cell populations. While plate-based BH3 profiling has been useful for understanding MCL biology from a basic science standpoint, iBH3 profiling can be used to characterize the response of patient samples to mimetic peptides, and this information can be used to predict a patient's response to chemotherapies before any treatments are administered. This has the potential to improve clinical outcomes of MCL patients through a personalized medicine approach.

# Appendix

1) Results from single agent studies with the PRMT5 inhibitor PRT-382 in nine MCL cell lines. IC50s were determined on day nine with viability measured with annexin V/PI staining and flow cytometry.

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